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Investigation of Hydrogen Sulphide Production in the Female Rat Reproductive tract

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Investigation of Hydrogen Sulphide Production in the Female Rat Reproductive tract

By

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Abstract

Premature labour is defined as uterine contractions with sufficient intensity, duration and frequency to produce progressive cervical effacement and dilatation before 37 weeks of gestation (Carson, 2004). It is one of the leading complications in obstetrics. There is evidence to suggest that hydrogen sulphide is involved in pregnancy and labour. Research has previously shown that H_2S can be produced by homogenates of rat and human placenta, fetal membranes and pregnant uterus and that H_2S donors, L-cysteine and sodium hydrosulfide, relax pregnant rat uterine smooth muscle in vitro. The aim of the present study was to investigate the production of H_2S by isolated cells of the rat placenta, fetal membranes and pregnant myometrium in culture. Cell culture techniques were performed to disperse Chorio-decidual cells from placenta, amnion/chorion/decidual cells from fetal membranes and myometrial smooth muscle. Cells were exposed to 1 mM L-cysteine for 48 hours and evolved H_2S was trapped in 1% zinc acetate solution. The resulting sulphide was measured using a standard methylene blue assay and expressed as nM H_2S produced per min per 10^5 cells (mean ± SD). The effect of exposing the cultured cells from the fetal membranes to 1 µg/ml lipopolysaccharide (LPS) on the production of H_2S was also investigated. Data were analysed using ANOVA or independent samples T test, as appropriate. Rat myometrial smooth muscle cells have a H_2S mean production rate of 3.63±1.21nM/min/10^5 cells (n=5), Chorio-decidual cells from the placenta have a H_2S mean production rate of 45.53± 12.61nM/min/10^5 cells (n=6) and cells from the fetal membranes have a H_2S mean production rate of 1.79±0.62nM/min/10^5 cells (n=5). Chorio-decidual cells from the placenta had a significantly ($P<0.001$) higher mean production rate of H_2S, in comparison to myometrial smooth muscle cells and cells from the fetal membranes. The results show that rat chorion, decidual, amnion and myometrial smooth muscle cells can produce H_2S in culture. Chorio-decidual cells from the placenta had the highest production rate of H_2S, which could be involved in vasodilation of placental blood vessels or maintaining myometrial quiescence. H_2S production from cultured fetal membrane cells did not increase with the addition of LPS suggesting that H_2S probably does not act as an inflammatory mediator in this model of intrauterine infection. The H_2S system requires further research and could be a future target for intervention in pregnancy and labour.

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List of Abbreviations (alphabetical order)

CBS	Cystathionine β-synthase
CO	Carbon monoxide
CSE	Cystathionine γ-lyase
H_2S	Hydrogen Sulphide
LPS	lipopolysaccharide
NaHS	Sodium hydrosulphide
NO	Nitric Oxide
PAG	DL-propargylglycine

Chapter One: Introduction

1.1: Human Pregnancy

Human gestation is normally for a period of 37- 42 weeks. The processes in human pregnancy from conception to delivery are not fully understood. Implantation of the embryo to the uterus wall occurs in the third week of pregnancy. There are various organs within the female reproductive system that are involved in pregnancy are: the uterus, the placenta, the fetal membranes and the umbilical cord.

Figure 1.1 Pregnant Human Uterus

Source: https://my.inova.com/public/healthresearch/content_display_full.cfm?doc_id=P02863

1.2: Human Uterus

The human uterus is a hollow, thick and muscular walled organ found between the urinary bladder and the rectum, and it is completely within the pelvis. The anatomical structure of the uterus shows that it comprises of the body (including the fundus) and the cervix. The fundus is located at the upper part of the body of the uterus connecting it to the ovaries via the Fallopian tube. Oocytes are stored in the ovaries and one is usually released every month. The oocyte becomes embedded in the uterus wall if it is fertilized. During pregnancy the uterus changes shape and size to cope with the development of the embryo and finally fetus until parturition where it returns almost to its former condition.

FIG.1.2 HAS BEEN REMOVED FOR COPYRIGHT REASONS.

The largest part of the uterus is the body. The thick muscular wall of the uterus body consists of three layers. The interior layer is the endometrium that functions as a inner glandular mucosal lining for the uterus (Prekumar, 2004). It undergoes changes with the menstrual cycle. The middle layer is the myometrium which is the muscular structure of the uterus wall. It is made up of smooth muscle arranged in longitudinal, oblique and circular layers (Prekumar, 2004). The outer layer is the perimetrium which is a thin layer composed of mesothelium and connective tissue. The cervix is the narrowing, lower part of the uterus which protrudes into the vagina.

1.3: Placenta

The placenta is an organ fasten to the lining of the womb during pregnancy. It is formed at the site of implantation of the embryo in the uterus via the uterine mucosa. It is a hemochorial villous multifunctional organ which is involved in the growth and development

of the fetus and it is therefore essential during pregnancy. It is involved in the exchange of gases and nutrients between the maternal and fetal systems and also the elimination of waste products from the fetus (Vause and Saroya, 2005). Vause and Saroya (2005) states that the placenta is an important site of hormone production.

Fig. 1.3 OF THE MATURE HUMAN PLACENTA HAS BEEN REMOVED FOR COPYRIGHT REASONS.

The mature human placenta is about 20cm in diameter and 3cm in thickness and is disc shaped. The developing embryo (blastocyst) has an outer ectodermal cellular layer called the trophoblast and the inner cell mass called the embryoblast. The trophoblast forms the interface between the uterine tissues and those of the implanted embryo. The trophoblast quickly differentiates into the syncytiotrophoblast and the cytotrophoblast. The syncytiotrophoblast is a multicellular mass while the cytotrophoblast is a mononuclear mitotically active layer (Blackburn, 2007). The chorionic villi are formed from the cytotrophoblast. This forms the villous system consisting of trabeculae and lacunae (vacuoles) which extend through the syncytiotrophoblast. Surrounding this system is the primary chorionic plate (towards the embryoblast) and the trophoblastic shell (towards the endometrium). Some of the villi are attached to the trophoblastic shell and are called anchoring villi. The lacunae system expands continuously to become the intervillous space. The maternal circulation is established by cells from the trophoblastic shell which move into the maternal endometrial vessels allowing maternal blood to enter. The fetus is connected to the placenta via the umbilical cord. It contains two arteries and a vein surrounded by a substance which contains collagen, muscle, and mucopolysaccharide (Polin et al, 2004). This substance that surronds the umbilical vessel within the umbilical

cord is called Wharton's jelly (Blackburn, 2007). The umbilical cord grows to a maximum average length of 55 to 60cm at 30weeks of pregnancy.

1.4: Fetal Membranes

The fetal membranes are essential for maintaining pregnancy. They play various roles including protecting the fetus against infection, fetal development and progression of pregnancy (Gude et al, 2004). The fetus is contained within the fetal membranes until the early stages of labour when they rupture . The fetal membranes are composed of the outer membrane which is known as the decidua, the middle membrane known as the chorion and the inner layer known as the amnionThe chorion comprises the cellular layer consisting of the fibroblast network (thin and present in early prgnancy), recticular layer (the major part of the chorion), a pseudo-basement membrane (acting as an anchor for the fibroblast) and the trophoblast cell layer containing 2 to 10 layers of trophoblast cells (Bourne, 2006).

The amnion is comprised of five layers, namely the epithelium (in direct contact with the amniotic fluid), the compact layer, the fibroblast layer, the intermediate (spongy) layer and the basement membrane (connective tissue) (Gude et al, 2004). The amniotic epithelium encloses the amniotic fluid.

1.5: Uterine Quiescence

Human gestation is normally for a period of 37- 42 weeks. The control of myometrial contractility is an important feature of pregnancy and parturition. The myometrium is maintained in a relaxed state during pregnancy to accommodate fetal growth; however the mechanism underlying uterine quiescence is not fully understood.

Uterine quiescence also known as myometrial quiescence is the relaxed state of the uterus during pregnancy up to labour. Carvajal et al (2009) suggests that quiescence is

characterised by profound myometrial insensitivity to agents that normally stimulate the uterus.

The maintenance of uterine quiescence is not fully understood however it can be concluded that it is maintained by a balance of various controlling factors which include hormones and substances that suppress the contractility of the myometrium. During labour the conditions in the myometrium favour stimulation and less of inhibition.

Fetal membranes produce one or more factors (e.g. nitric oxide) that act to inhibit myometrial contractions and therefore are mediators of quiescence by opening Ca^{2+} activated K^+ channels (BK_{Ca}) (Carvajal et al, 2006).

The myometrial cAMP pathway is active during pregnancy and declines significantly as labour approaches. According to Carvajal et al (2009) Gαs, a heterotrimeric G-protein is the most prominent during pregnancy. Its expression and coupling to adenylate cyclase promotes the increased activity of the cAMP pathway and it is said to be increased during pregnancy, which maintains myometrial relaxation. The cGMP pathway also increases during quiescence and declines before the onset of labour, it also inhibits myometrial contraction leading to the suggestion that it is involved in quiescence.

Nitric oxide, which is produced in intrauterine tissues, has been implicated in uterine relaxation. Yallampalli et al (1998) suggested that NO production occurs in parallel to changes in expression of NOS II (iNOS). NOS II (iNOS) expression is upregulated by progesterone, which also up-regulates the production of NO and increases the relaxant effects of NO. A decrease in NO production or its activity may therefore contribute to initiation of parturition (Seyffarth et al., 2004). Uterine quiescence during pregnancy and increased contractility during labour are maintained partially by the NO system. The NO

system interacts with prostaglandins and steroid hormones such as progesterone to mediate their effects on uterine contractility.

1.6: Mechanism of Human parturition

Labour onset and its progression are controlled by a complex arrangement of many factors found in maternal, fetal, and placental tissue. These factors act as signals to either maintain a single essential labour-related event and they include prostaglandins, cortisol, progesterone and oxytocin. It is known that the onset of labour is an inflammatory event. These factors also interact with cytokines in a cascade that has multiple effects. Cytokines play a role in the inflammatory process. Steinborn et al (1999) suggested that inflammation is linked to spontaneous preterm onset of labour.

The beginning of labour is usually signalled by regular uterine contractions and changes in the cervix. The cervix consists of stiff, fibrous tissue during pregnancy in order to retain the uterine contents. Prior to delivery the cervix undergoes biochemical changes, termed ripening, usually from about the 34th week onwards. The cytokine IL-1 stimulates the synthesis of matrix metalloproteinases (MMPs) which are implicated in cervical tissue changes associated with labour as well as in the weakening of the membranes in the region overlying the cervix prior to rupture (Farinam and Winkelman, 2005).

With the onset of uterine contractions, white blood cells migrate into the uterine organs and release pro-inflammatory cytokines. Pro-inflammatory cytokines are also directly produced from labouring uterus, placental and fetal tissues. Changes in the levels of cytokines IL-1, IL-6, IL-8 and TNF have been implicated in the onset and progression of

pre-term labour. Steinborn et al (1999) stated that cytokines initiate various events which include production of prostaglandins, induction of COX2, as well as other factors necessary for labour.

In myometrial contraction, myosin light chain kinase is activated by Ca^{2+}. There is a correlation between increased actomyosin ATPase activity, contraction and phosphorylation of myosin light chain. The tension development of the smooth muscles in the uterus is decreased when myosin light chain phosphorylation is decreased and vice versa.

Myosin has enzymatic activity and is a structural protein which is laid down in thick myofilaments. Its enzymatic activity involves the conversion of ATP into motion/force generation during contraction. It is composed of two heavy chains of 200,000 KDa each and two light chains of about 20,000 and 15,000 KDa. Myosin has a globular head which carries three important sites. The first site is the ATPase site where ATP is hydrolyzed. The second site is the site where it interacts with actin and the third site is the 20,000 KDa light chains which produces the key element of contractile regulation through its phosphorylation. Calcium is vital in contractility via the enzyme myosin light chain kinase which regulates the phosphorylation and de-phosphorylation of 20,000 KDa light chain of myosin enabling the actin and myosin interaction. If myosin light chains are not phosphorylated then the interaction between myosin and actin will not occur.

Oxytocin induces uterine contractions, however it is controversial whether oxytocin in the blood or that released locally is important in human labour.

1.7: Premature Labour

Premature labour is defined as uterine contractions with sufficient intensity, duration and frequency to produce progressive cervical effacement and dilatation before 37 weeks of gestation (Carson, 2004). It is one of the leading complications in obstetrics. In the UK it is said to affect 10% of all pregnancy and accounts for 60% of neonatal deaths. It is also the said to be the major cause of childhood disability (Gullam et al, 2005).

The causes of premature labour are being constantly researched yet they remain unidentified. There have been multiple factors which have been associated with premature labour including fetal development, multiple pregnancies, previous premature deliveries, cervical incompetence, the spontaneous rupture of the membranes (PROM) and intrauterine infections. Although these factors have a bearing on premature labour it is still impossible to predict which women will go into premature or preterm labour.

The detection of premature labour is an ongoing process however the possibility of biochemical and clinical makers are being studied. In hospitals premature labour is assessed using clinical criteria (Carbonne, 2004). According to Carbonne (2004) this method of clinical assessment of uterine contractions and of cervical changes is highly subjective; therefore sensitive methods of detecting patients who are at risk of preterm birth are needed to prevent unnecessary treatment or hospital admissions.

The measurement of electromyography (EMG) is also a means of premature labour detection. There are two methods by which uterine EMG can be measured; through the uterus using needle electrodes or via abdominal surface electrodes. This method measures uterine contractility using electrical activity allowing for evaluation of the state of

the uterus for delivery. The measurement of collagen breakdown using light induced fluorescence is another technique for detection of premature labour. The cervix contains collagen which is fluorescent. Collagen emits light at a typical wavelength of 390 nm. During pregnancy (before labour) there is a higher concentration of collagen in the cervix and therefore a higher light-induced fluorescence level is measured. As the cervix ripens, before and during labour, the collagen breaks down leading to a lower measurement of light-induced fluorescence.

Fetal fibronectin is a biochemical marker which has been seen to have strong associations with premature labour. Fibronectin is an extracellular matrix glycoprotein produced by the extravillious cytotrophoblast. It is a “glue-like” protein that bonds the developing baby to the uterus. During 22 and 37 weeks of gestation, if the level of fetal fibronectin in cervicovaginal secretions is greater than 50ng/ml this indicates a higher risk of preterm labour (Petraglia et al, 2007). The negative predictive value of fibronectin is useful in identifying patients who are not at risk for preterm birth. Rizzo et al. (2006) suggests that joint use of the fetal fibronectin assay and the ultrasound cervical index improves the diagnostic efficiency in preterm labour.

The prevention and treatment of premature labour is still being researched and remains a difficult area. The agents that are used worldwide currently to prevent premature labour include β-agonist drugs (ritodrine, terbutaline, salbutamol), oxytocin receptor antagonist (atosiban), magnesium sulphate, calcium channel blockers (nifedipine), nitric oxide donors and prostaglandin synthesis inhibitors. The β-agonist drugs are used to reduce the intensity of preterm labour however their effects last only between 24-48hours (Carson, 2004). It may be effective for some patients however it has been known to have

undesirable side effects. Atosiban is a competitive oxytocin receptor antagonist and it is the only oxytocin-antagonist licensed for use in preterm labour. With the inhibition of the oxytocin receptor there is a reduction in extracellular calcium influx and release of calcium from intracellular stores which leads to inhibition of contractility. Atosiban has fewer side effects than β-agonists and is apparently more effective in treating preterm labour (Gullam et al, 2005).

Nifedipine is the most commonly used calcium channel blocker for tocolysis. Calcium channel blockers are smooth muscle relaxants which are non-specific and act by inhibiting the intracellular influx of calcium ions. There have been varying reports on the side effects of nifidipine from no side effects at all to adverse fetal and maternal side effects.

Magnesium sulphate is a preterm labour management drug used widely but predominantly in the United States. It has been met with major opposition in the United Kingdom, Europe and Australia as the evidence supporting its use are controversial as it shows no tocolytic efficacy. In high doses magnesium sulphate is implicated in possible contribution to infant mortality (Pryde et al, 2001). Gasotransmitters are a new look into possible tocolytic agents. Nitric oxide has been found to relax uterine smooth muscle contractility. Leszczynska-Gorzelak et al, 2001 conducted a clinical trial using nitroglycerine as a donor of nitric oxide. It was shown as an effective and safe tocolytic drug, however it is suggested that more research needs to be carried out (Leszczynska-Gorzelak et al, 2001).

Although there are various drugs that seem to have an effect on prevention and treatment of preterm labour none of them have high positive results with low side effects. More research needs to be done to find tocolytic therapies that are effective in preterm labour with reduced side effects allowing for fetal maturity.

1.8: Hydrogen Sulphide

The mechanism of the onset of Labour within humans is not fully known. However there is evidence to suggest that hydrogen sulphide is involved in this process. Hydrogen sulphide is a gas signalling molecule. The role of carbon monoxide and nitric oxide as gas signalling molecules within mammalian tissue has been extensively researched. Recently the importance of hydrogen sulphide in mammalian tissues as a gas signalling molecule has come to light. Although it is still early stages a lot of research is dedicated to better understanding the pharmacological, physiological and pathological roles of this gas signalling molecule and understanding how it reacts with the other gas signalling molecules.

Hydrogen Sulphide is a flammable, colourless gas that is highly present in the atmosphere. It is easily noticeable as it has a distinctive rotten-egg smell. H_2S is a hazardous released from various sources into the atmosphere. For example it is released from swamps, marshes, volcanic gases and also during natural gas production and sewage treatment. Most of the work done on Hydrogen sulphide has focused on its toxicity. In high dosages H_2S is lethal. The presence of Hydrogen sulphide has been investigated within mammalian tissues. It has been discovered in the brain, smooth muscles, vascular tissue, uterus and other tissues.

H_2S production within mammalian tissues is due mainly to two enzymes: cystathionine β synthase (CBS) and cystathionine γ Lyase (CSE) using L-cysteine as the main substrate (Stipanuk and Beck, 1982). CBS and CSE have been found to be expressed in various mammalian tissues. CSE is found in the liver, kidney, small intestine, stomach (Ishii et al

2004) and blood vessels (Hosoki et al 1997) while CBS occurs abundantly in the brain (Abe and Kimura 1996).

The first biologically significant role of H_2S was discovered in 1996 by Abe and Kimura. They discovered high concentrations of H_2S in the brain and found that it acts as a neuromodulator or neurotransmitter. In 1997 Hosoki et al. discovered that H_2S producing enzymes are present in the ileum, portal vein and thoracic aorta suggesting that H_2S was biologically significant in the tissues. It was concluded that H_2S acts smooth muscles as a relaxant. In 2001 Zhao et al. concluded that H_2S has a cardiovascular effect of relaxation and reduces blood pressure in vivo. H_2S generation in the pancreas helps to regulate insulin release. There is evidence to suggest that H_2S acts as an inflammatory mediator. H_2S is produced in intrauterine tissues (uterus, fetal membranes and human placenta) and it acts as a relaxant for pregnant rat uterus. Low levels of H_2S were found to cause a dose-dependent increase in the duration of labour in rats suggesting that it might have an effect in pregnancy (Hayden *et al*., 1990).

1.9: Hydrogen Sulphide as a neuromodulator or neurotransmitter

It was discovered that H_2S is present at relatively high concentrations within the brain (Abe and Kimura, 1996) bringing about the conclusion that it has physiological function. H_2S – producing enzyme cystathionine β–synthase (CBS) is found to be highly expressed in the brain specifically the hippocampus and cerebellum. Furthermore it has been observed that the concentration of H_2S within the brain is in the range of 50 to 160μM. From the observations research was done using CBS inhibitors and activators to discover if it was

the major mechanism of production of H_2S within the brain. The conclusion of this research is that CBS is the major H_2S –producing enzyme in the brain.

The roles of H_2S in the brain are not yet fully understood but it is involved in modulation of synaptic activities regulated by steroids and neurotransmitters. Physiological concentrations of H_2S induces long-term potential (LTP) at active synapses and this requires the activation of NMDA receptors. NMDA receptor antagonist, 2-amino-S-phosphonovalerate, blocks the LTP previously seen to be induced by NaHS (H_2S donor) with a weak tetanic stimulation. These observations suggest that H_2S functions as a neuromodulator activating NMDA receptors. Although this seems to be the main mechanism for H_2S action research is still ongoing to discover if there are any pathways to which H_2S acts as a neuromodulator on inactive synapses.

There are a number of pathways involved in the regulation of H_2S in the brain. Ca^{2+} - and calmodulin-mediated pathway are implicated in the regulation of H_2S. Ca^{2+} /calmodulin mediate the activity of CBS. Although the actual mechanism of action is unknown it is suggest that there is a binding site for Ca^{2+}/calmodulin on CBS which activates it once attached. CBS activity is regulated by S-adenosyl-L-methionine (SAM) (Eto et al., 2002). The regulation of H_2S has been linked to testosterone. There is a difference in the amount of H_2S in the brain of male mice, which contains more testosterone, in comparison to female mice. When male mice are castrated, reducing the testosterone, the amount of H_2S and SAM reduced. Similarly, when female mice are given an injection of testosterone the amount of H_2S and SAM are increased. However the difference in H_2S between male and female mice is much less than the difference in testosterone leading to the conclusion that

testosterone only partly regulates H_2S. It is suggested that testosterone induces SAM which regulates H_2S downstream.

1.10: Hydrogen Sulphide as an inflammatory mediator

Hydrogen Sulphide's role in inflammation is still in the early stages of research therefore not much is known about its role. Li, Bhatia and Moore (2006) suggest that H_2S is a mediator of inflammation. In acute pancreatitis H_2S may be acting as an inducer of neurogenic inflammation which support to the role of H_2S as an inflammatory mediator. In this condition rats treated with D,L-propargylglycine (PAG) had a significant attenuation of hyperamylasemia, acinar cell injury/necrosis, pancreatic myeloperoxidase oxidase activity and a histological evidence of diminished pancreatic injury. This suggests a reduction in the severity of pancreatitis. DL-propargylglycine inhibits CSE and reduces inflammation (Bhatia et al., 2005)

H_2S induces the generation of pro-inflammatory cytokines through the upregulation of ERK-NF-$_k$B in human monocytes (Zhi et al., 2007). H_2S-releasing dithiolthione moiety attached to diclofenac is the composition of S-diclofenac phenyl ester. S-diclofenac inhibits inflammation in endotoxic shock showing that H_2S has an anti-inflammatory effect.

Addition of NaHS (H_2S donor) into peripheral tissue causes an increased sensitivity to pain in rat hindpaw. Cystathionine gamma lyase is responsible for endogenous H_2S in peripheral tissue as inhibition of this enzyme eliminates the hyperalgesia. H_2S sensitizes T-type Ca^{2+} channels by its reducing effect leading to hyperalgesia (Kawabata et al, 2007).

1.11: Hydrogen Sulphide as a vasorelaxant

There have been a number of studies done on the role of the H_2S as a vasorelaxant. The initial research carried out by Hosoki, Matsuki and Kimura (1997) was to discover the possible role of H_2S on smooth muscle. The H_2S-producing enzyme cystathionine γ-lyase is expressed in the ileum, portal vein and thoracic aorta and also cystathionine β-synthase is expressed ileum only leading to research to measure H_2S within these tissues. There is a significant amount of H_2S found within the tissues suggesting that it has a physiological role in the smooth muscle. It was shown that the effect of H_2S on smooth muscle within these tissues was dose dependent relaxation.

The vasorelaxant effect of H_2S is due to its direct interaction with smooth muscle cells. CSE mRNA has been located in vascular smooth muscle cells but not in endothelial layer of rat aortic wall (Zhao et al, 2001). The observation that H_2S directly interacts with smooth muscle cells was reached by Zhao and Wang (2002) based on the fact that H_2S still relaxed vascular tissues after endothelium removal and also on the failure of removal of nerve supply to the tissue to alter H_2S effect. Further research has suggested that vasorelaxant effect of H_2S is endothelium-dependent, contradicting previous notions suggesting the opposite, but this dependency is tissue specific. The H_2S-induced relaxation is significantly reduced in mesenteric artery bed (MAB) when endothelium is removed but it is only slightly weakened in rat aortic tissue.

The H_2S relaxation of blood vessels occurs through the opening of K_{ATP} channels and the H_2S–induced vasorelaxation is dependent on extracellular calcium. H_2S may reduce the extracellular calcium entry and relax tissues. The mechanism for this may be through membrane hyperpolarisation occurring when K_{ATP} channels open which could close voltage-gated Ca^{2+} channels (Zhao and Wang, 2002). Alternatively there could be a direct

interaction between voltage-gated Ca^{2+} channels in vascular smooth muscle cells and H_2S.

However another mechanism for action of H_2S relaxation other than its action on K_{ATP} channels has been discovered. NaHS decreases pH_i in a concentration-dependent manner in vascular tissue suggesting that endogenous H_2S has a role in regulating pH_i. In vascular smooth muscle a decrease in pH_i relaxes vascular vessels. H_2S decreases pH_i by enhancing the activity of an acid loader Cl^-/HCO_3^- exchanger in smooth muscle cells (Lee et al., 2007). It is known that intracellular acidification directly stimulates K_{ATP} channels (Wang et al., 2003). Therefore there may be a relationship between H_2S opening of K_{ATP} channels and its role in decreasing intracellular pH.

It has been observed that H_2S relaxes the smooth muscle in synergy with nitric oxide (Hosoki et al 1997). This observation has been supported by Zhao et al (2001) with the discovery that NO donors appear to increase H_2S in vascular tissues. This increase is due to NO increasing the expression and stimulation of the activity of H_2S producing enzyme CSE. CSE is stimulated by c-GMP-dependent protein kinase whose activity is increased by NO. Blocking c-GMP-dependent protein kinase removes the NO-induced increase in H_2S level in vascular tissue (Zhao et al., 2003).

1.12: Hydrogen Sulphide and cardiovascular system

Research was conducted by Zhao et al (2001) demonstrating the cardiovascular effects of H_2S. It showed that intravenous injection of H_2S provokes a decrease in mean arterial blood pressure. The effects of H_2S are due to its action on vascular smooth muscle as there is no significant change in heart rate. There is a reduction in H_2S produced when cystathionine gamma-lyase (CSE) is inhibited leading to a reduction in the vasorelaxant effect of H_2S. This leads to elevated peripheral vascular resistance and increased BP. The relaxation occurs through the opening of K_{ATP} channels.

The endogenous production of H_2S in the posterior hypothalamus by cystathionine beta-synthase (CBS) may contribute to central regulation of arterial blood pressure. It was shown that infusion of NaHS and the CBS activator; s-adenosyl-l-methionine (SAM), into the hypothalamus increases the mean arterial blood pressure while infusion with hydroxylamine (HA), a CBS inhibitor, had the opposite effect. K_{ATP} channel-dependent mechanism seems to mediate this effect (Dawe et al., 2007).

Recently it has been demonstrated that H_2S could contribute to cardio-protection during ischemic reperfusion or hypoxia (Geng et al 2004). This could be as a result of mediation of K_{ATP} channel pathway by H_2S produced by heart tissue thereby acting as a physiological cardiac function regulator. The cardioprotective effect of H_2S also involves the preservation of mitochondrial structure and function after ischemia-reperfusion (Elrod et al., 2007). It also reduces human vascular smooth muscle cell proliferation by increasing the phosphorylation of cardiac extracellular-signal-regulated kinase (ERK) and PI3K/Akt pathways. H_2S negatively affects the force of muscle contraction and could play a role as a regulatory gas signal molecule in the heart (Geng et al., 2004).

1.13: Other roles of Hydrogen Sulphide

H_2S-producing enzymes, CBS and CSE, are found in relatively large amounts in the pancreatic islets. Pancreatic H_2S generation might be involved in the regulation of insulin release under physiological conditions. NaHS (H_2S donor) and L-cysteine inhibits the glucose induced insulin release in pancreatic islets. The levels of endogenous H_2S are lowered with an increase in extracellular glucose (Yang et al., 2005). H_2S regulates K_{ATP} channels which are known to regulate the function of insulin-secreting pancreatic β cells.

HS^- ion has a pro-survival effect. Rinaldi et al (2006) discovered that HS^- ions delay the onset of apotosis in polymorphonuclear neutrophils by inhibiting Caspase-3 cleavage and p38 MAPK phosphorylation. However, in lymphocytes the effect is the reverse. This pro-survival activity of HS^- ion in neurophils could accelerate the resolution of inflammatory processes and prevents the occurrence of new ones.

NaHS causes strong relaxation in isolated mouse main bronchus but only slightly relaxes guinea-pig bronchus. However the mechanism underlying this relaxation is still unclear. It has been shown that this relaxation is not induced by K^+_{ATP} channels or the NO-cGMP pathway. In guinea-pig airways H_2S provokes tachykinin-mediated neurogenic inflammatory responses. This effect is said to be mediated by the activation of transient receptor potential vanilloid 1 (TRPV1) receptors on sensory nerve endings.

There is a reduction in the vasoconstriction caused by norepinephrine when liver tissue is exposed to H_2S and L-cysteine. H_2S is a physiologically relevant mediator in intrahepatic resistance. The hypotensive effect of H_2S is mediated through the opening of K_{ATP} channels which causes the relaxation of resistance blood vessels of the hepatic

microcirculation. In liver with cirrhosis there is a reduction in the expression and activity of cystathionine gamma lyase leading to a reduction in hepatic H_2S production. The mechanism of reduction of CSE activity in cirrhosis is unknown however the involvement of H_2S could help in creating new drugs for the condition.

L-cysteine and NaHS (a H_2S donor) relaxed pregnant rat uterus in vitro (Sidhu *et al*., 2001). The expression of CBS and CSE has been detected in rat intrauterine tissues: uterus, placenta and fetal membranes. H_2S is produced in the intrauterine tissue (rat uterus, placenta, fetal membranes and also human placenta) with the addition of L-cysteine (Patel et al. 2008). The production of H_2S in rat fetal membranes is increased by nitric oxide. The production of H_2S was significantly elevated under hypoxic conditions in human placenta, rat liver, uterus and fetal membranes. In fetal membrane, rat uterus and human placenta there is an increase in endogenous production of H_2S under hypoxic conditions. H_2S may have a role as an inflammatory mediator in pregnancy and could be specifically associated with placental pre-eclampsia (Patel et al, 2008).

AIMS

The aim of this project is to investigate which cells of the rat uterus, placenta and fetal membranes produce hydrogen sulphide, from donors, in culture.

The objectives are to critically review literature relating to hydrogen sulphide production and action in the body, to extract cells from the uterus, placenta and fetal membranes of pregnant rats and maintain them in culture and measure hydrogen sulphide production by these cells in culture. There will also be an investigation into the factors that affect hydrogen sulphide production by these cells in culture.

Chapter Two: Materials and Methods

Apparatus

Scalpel, scissors, laminar flow cabinet, beakers, spray bottles, centrifuge tubes, 200 µm pore nylon mesh, filter funnel, centrifuge, culture flasks, cell scraper, Pasteur pipettes, auto pipettes, sterile tips, Petri dish, lab coat, gloves, trapping tube, test tubes, filter paper.

All consumables were obtained from Sigma (Poole, UK).

Chemicals:

70% Industrial methylated spirit (IMS), VirKon, HAMS F-12, DNase, Trypsin, Collagenase, Fetal Bovine Serum, Penicillin Streptomycin (PenStrep), Nystatin, Zinc acetate, N, N-dimethyl-p-phenyldiamine sulphate, ferric chloride ($FeCl_3$), Sodium hydrosulphide (NaHS), Hydrochloric acid (HCl)

All of the chemicals used were obtained from Sigma-Aldrich (Poole, UK)

2.1: Cell culture technique

The pregnant rats at 19 days of gestation were used as a model for human pregnancy. The uterus, placenta and fetal membrane were obtained from the rats. The tissues were minced into approximately 2mm fragments using sterile scissors and blades. The tissues were transferred to sterile centrifuge tubes and digested in 10ml sterile HAMS F-12 medium containing 0.1% DNAase and 0.1% trypsin for 30minutes at 37^oC with shaking. 0.1% collagenase in HAMS F-12 medium was added and digested for a further 30minutes.

The mixture was then filtered through sterile 200 µM pore nylon mesh. The mixture was then centrifuged at 500g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 10ml culture medium containing 10% fetal bovine serum, 1% Nystatin and 2% PenStrep. The resuspended cells were then centrifuged again (called washing) and the supernatant removed. The cells were re-suspended in 10 ml complete medium as above. A cell count using a haemocytometer slide was performed and also a viability test using trypan blue. Trypan blue stains the nuclei of non-viable cells while viable cells remained unstained. This staining was seen under the microscope.

The cell suspensions were placed into a culture flask and incubated for 48 h prior to use, so that they could recover from the digestion. After the first incubation the complete medium were replaced then 1mM of L-cysteine were added. A tube containing 1% zinc acetate was inserted into the flask. The zinc acetate traps any hydrogen sulphide gas produced by converting it into zinc sulphide. The cells were incubated for a further 48 hours. Any zinc sulphide formed in the trapping tube was measured using the standard methylene blue assay.

2.2: Methylene blue assay

The methylene blue assay is a colourimetric assay designed to measure the concentration of sulphide ions. Sodium hydrosulphide (NaHS) was used as a H_2S donor to produce calibration standards.

20mM N, N-dimethyl-p-phenyldiamine sulphate was prepared in 7.2M HCl. 30mM ferric chloride was prepared in 1.2M HCl. A 10mM stock solution of NaHS was prepared in 50ml of distilled water.

NaHS (10mM) was diluted to 5mM, by taking 500µl of the stock solution and diluting with 500µl distilled water. The 5mM NaHS solution was then diluted to make a 2.5mM NaHS solution, this was continued to produce the calibration standards (as seen in table below)

Standard concentrations of NaHS (mM)

Test Tube	0	1	2	3	4	5	6	7	8	9	10	11
NaHS con. (mM)	0	0.005	0.01	0.02	0.039	0.078	0.156	0.313	0.625	1.25	2.5	5

3.5ml of distilled water was added to each calibration standard, followed by 400µl of 20mM N, N-dimethyl-p-phenyldiamine sulphate and 400µl 30mM ferric chloride. The calibrators were incubated at room temperature for 20minutes and the absorbance was measured at 670nm on Cecil 1010 spectrophotometer. A calibration curve was produced from the results.

The Methylene blue test involves the reaction of aqueous sulfide with N,N-dimethylphenyl-1,4-diamine in the presence of a small quantity of ferric ions which gives rise to a characteristic blue coloration. Quantification of the amount of Methylene blue formed can be easily achieved through UV spectroscopic methods.

2.3: Measurement of H_2S production in trapping tube

3.5ml of distilled water was added to the each trapping tube. 400µl of 20mM N, N-dimethyl-p-phenyldiamine sulphate and 400µl of 30mM ferric chloride was then added. The tubes were incubated at room temperature for 20minutes and the absorbance was measured at 670nm on Cecil 1010 spectrophotometer. Calibration standards were prepared using a 10mM stock solution of NaHS as described previously. A calibration curve for the NaHS standards was produced to calculate the concentration of hydrogen sulphide. Sulphide concentrations were used to calculate the production rate of hydrogen sulphide.

The baseline production of H_2S was investigated in smooth muscle cells from the myometrium, chorio-decidual cells from the placenta and amnion/chorion/decidual cells from the fetal membranes (n = 5 in each case).

Amnion cells from the fetal membranes were cultured in the presence of lipopolysaccharide (LPS) which will model an intra-uterine infection, and H_2S production were compared to baseline (n = 6).

2.4: Calculations

Cell count

X				X
		X		
X				X

Haemocytometer slide

Cell count: To get the approximate cell count for the solution we add the cell counts from all the cells marked X and Divide it by 5. It was then multiplied by 20,000.

The concentration of the H_2S production is converted to nM per min per 100,000cells. This is done by dividing the concentration mM divided the number of minutes in which the H_2S production is collected multiplied by 1,000,000 (conversion from mM to nM). It is then divided by the cell count for each solution.

$$= \frac{(\text{concentration in mM/minutes}) * 1{,}000{,}000}{\text{Cell count}/100{,}000}$$

2.5: Data Analysis

Using the calibration curves derived from the Methylene blue assay the concentration of the H_2S production is calculated for each membrane in nM per min 10^5 cells (mean ± SD). Data were analysed using ANOVA or independent samples T test to compare production of H_2S in the various tissue types and to compare if there is any significant difference between the H_2S production in fetal membranes exposed to 1 µg/ml lipopolysaccharide (LPS).

Chapter three: Results

After carrying out the cell culture technique as mentioned in the methodology section the cell count was calculated.

CELL COUNT		
Top Left	1	3
Top Right	0	0
Middle	0	1
Bottom left	4	0
Bottom right	1	0
Total	6	4
AVERAGE	5	

Cell count is then calculated as 5/5 * 20,000 = 20,000 per ml

= 200,000 per 10ml

3.1: Validation of Methylene Blue Assay

Methylene blue assay was validated using the dilution concentrations Table 3.1 below.

Test Tube	0	1	2	3	4	5	6	7	8	9
NaHS con. (mM)	0	0.005	0.01	0.02	0.039	0.078	0.156	0.313	0.625	1.25

Table 3.1: The dilution concentrations for calibration curve.

The absorbance of the standard listed in Table 3.1 was measured at 670nm on a Cecil 1010 spectrophotometer to produce calibration curve.

Concentration (mM)	Absorbance A	$x = \frac{y+0.0008}{1.6566}$
0.000	0.000	0
0.005	0.007	0.004227
0.010	0.015	0.009058

0.020	0.035	0.021135
0.039	0.054	0.032609
0.078	0.127	0.076691
0.156	0.242	0.146135
0.313	0.548	0.330918
0.625	1.034	0.624396
1.250	2.065	1.246981

Table 3.2: Absorbance of NaHS Calibration standards

The data recorded from the Methylene blue assay (e.g. Table 3.2) was inputted into an excel spreadsheet. XY Scatter graph was then generated and a Linear Trendline was plotted on the graph and the equation for the trendline was generated (e.g. Graph 3.1). This Linear regression equation was used to check the proximity in concentration to the calibration standards.

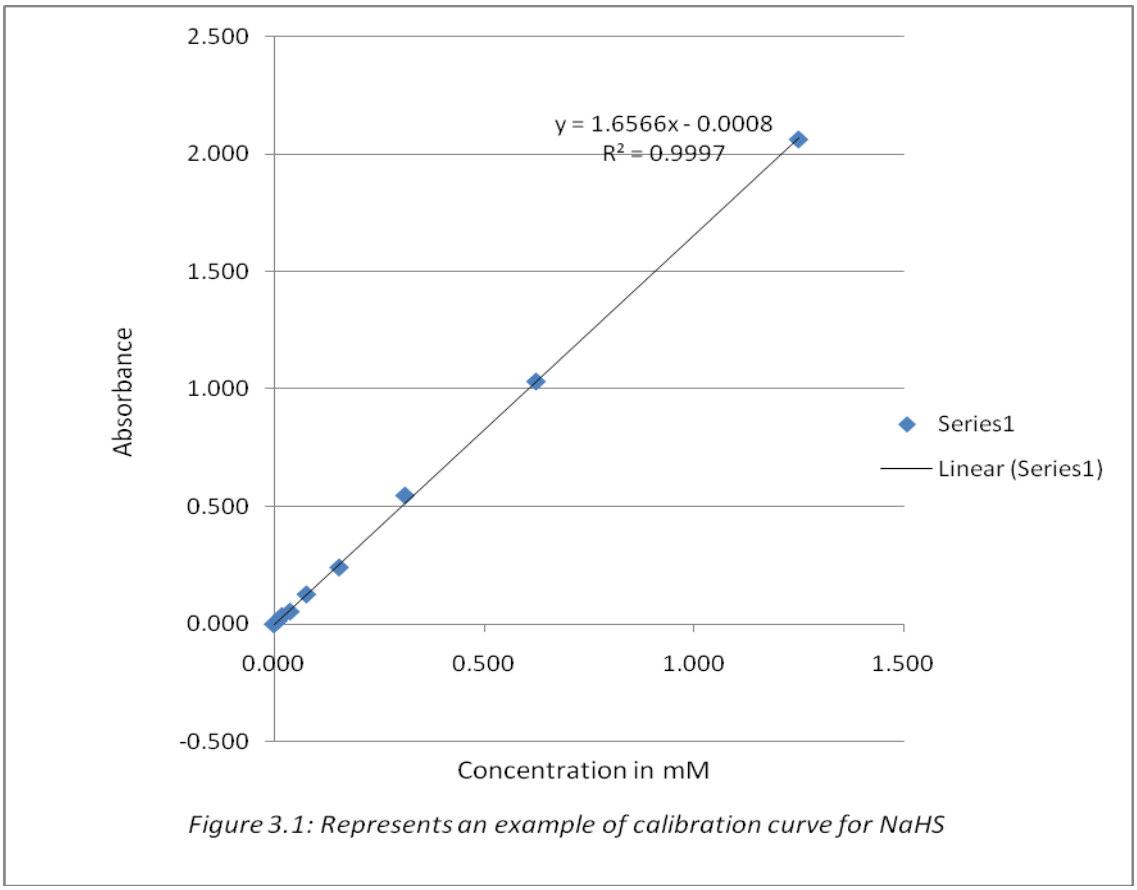

Figure 3.1: Represents an example of calibration curve for NaHS

The reproducibility of the Methylene blue was checked, the results verified.

3.2: The Endogenous Production of H_2S

The endogenous production rate of H_2S was calculated from the trapped sulphide concentration by using the following formula:

$$\frac{\text{(Concentration in mM/minutes)} * 1{,}000{,}000}{\text{Cell count/100,000}} = \text{Rate of } H_2S \text{ production nM/min/100,000cells}$$

3.3: Baseline Production of H_2S

The baseline production of H_2S was measured in different rat intrauterine tissues with 1 mM L-cysteine. The baseline production of H_2S is shown in tables 3.3, 3.4 and 3.5.

Date	Amplitude (A)	Concentration	Cell Count	Conc. in nM per min	Conc. in nM per min per 10^5cells
22-Feb	0.025	0.015	200,000	5.24	2.62
29-Feb	4.193	2.175	400,000	755.21	188.80
06-Mar	4.010	1.883	400,000	653.82	163.45
11-Mar	0.099	4.344	200,000	1508.33	754.17
13-Mar	1.181	5.272	400,000	1830.56	457.64
14-Mar	1.131	5.048	200,000	1752.78	876.39
17-Mar	1.139	5.024	360,000	1744.44	484.57
02-Apr	1.141	5.120	240,000	1777.78	740.74
08-Apr	0.033	0.022	240,000	7.64	3.18
09-Apr	0.026	0.018	110,000	6.25	5.68
18-Apr	0.022	0.019	220,000	6.60	3.00
24-Apr	0.019	0.021	200,000	7.29	3.65

Table 3.3: The baseline production of H_2S in smooth muscle cells from the myometrium. The values in red represent the infected cell cultures.

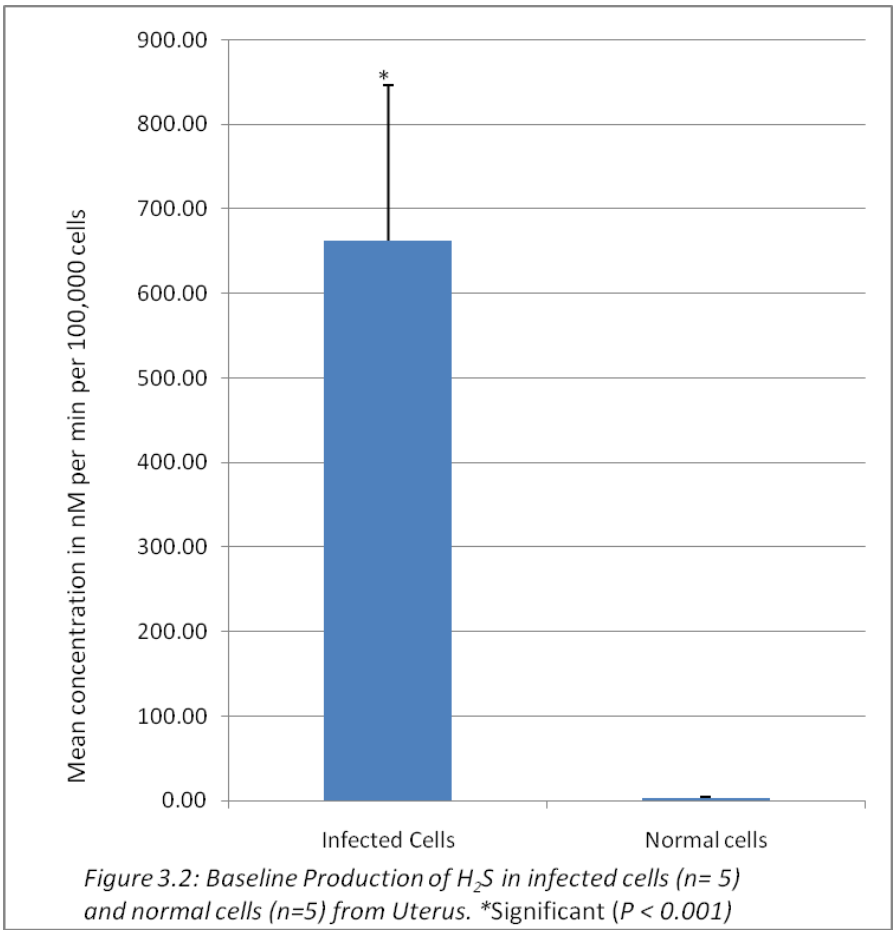

Figure 3.2: Baseline Production of H_2S in infected cells (n= 5) and normal cells (n=5) from Uterus. *Significant ($P < 0.001$)

Rat myometrial smooth muscle cells have a H_2S mean production rate of 3.63±1.21nM/min/10^5 cells (n=5) in normal cells and H_2S mean production rate of 662.70± 182.96nM/min/10^5 cells (n=5) in infected cells. Infected cells had a significantly (P<0.001) higher mean production rate of H_2S, in comparison normal cells.

Date	Amplitude (A)	Concentration	Cell Count	Conc. in nM per min	Conc. in nM per min per 10^5cells
01-May	1.481	2.012	200,000	698.61	349.31
30-Apr	0.773	5.360	200,000	1861.11	930.56
07-May	0.902	9.020	120,000	3131.94	2609.95
20-May	0.074	0.068	110,000	23.61	21.46
27-May	0.180	0.148	110,000	62.85	57.13
28-May	0.203	0.164	110,000	56.94	51.77
05-Jun	1.758	2.738	110,000	950.69	864.27
11-Jun	0.180	0.161	110,000	55.90	50.82
14-Jul	0.221	0.168	120,000	58.33	48.61
28-Jul	0.202	0.137	110,000	47.71	43.37
16-Jun	0.852	5.960	400,000	2069.44	517.36
18-Jun	0.845	5.900	300,000	2048.61	682.87

Table 3.4: The baseline production of H_2S in chorio-decidual cells from the placenta membrane. The values in red represent the infected cell cultures.

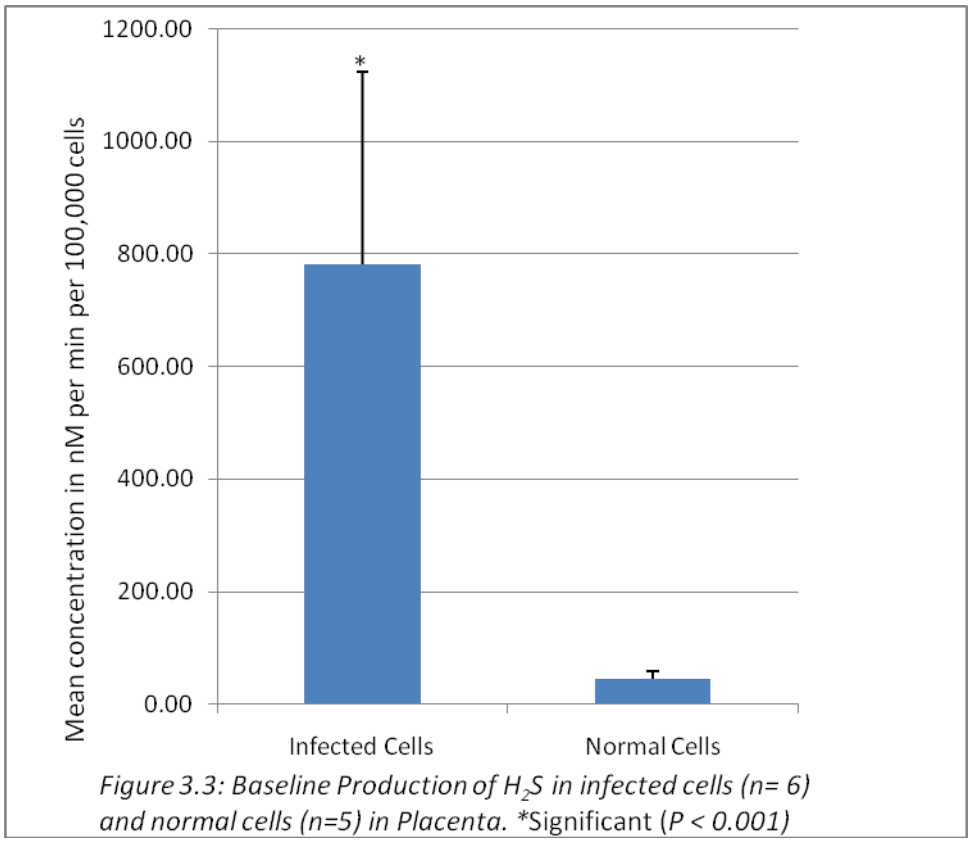

Figure 3.3: Baseline Production of H_2S in infected cells (n= 6) and normal cells (n=5) in Placenta. *Significant (*P < 0.001*)

Chorio-decidual cells from the placenta have a H_2S mean production rate of 45.53± 12.61nM/min/10^5 cells (n=6) in normal cells and H_2S mean production rate of 782.22± 342.59nM/min/10^5 cells (n=5) in infected cells. Infected cells had a significantly (P<0.001) higher mean production rate of H_2S, in comparison normal cells.

Date	Amplitude (A)	Concentration	Cell Count	Conc. in nM per min	Conc. in nM per min per 10^5cells
09-May	0.026	0.030	800,000	10.42	1.30
27-May	0.007	0.025	800,000	8.68	1.09
28-May	0.502	0.503	600,000	174.65	29.11
05-Jun	1.256	1.970	800,000	684.03	85.50
06-Jun	0.583	0.469	600,000	162.85	27.14
09-Jul	0.025	0.044	800,000	15.28	1.91
28-Jul	0.038	0.046	800,000	15.97	2.00
29-Jul	0.091	0.061	800,000	21.18	2.65

Table 3.5: The baseline production of H_2S in amnion/chorion/decidual cells from the fetal membranes. The values in red represent the infected cell cultures.

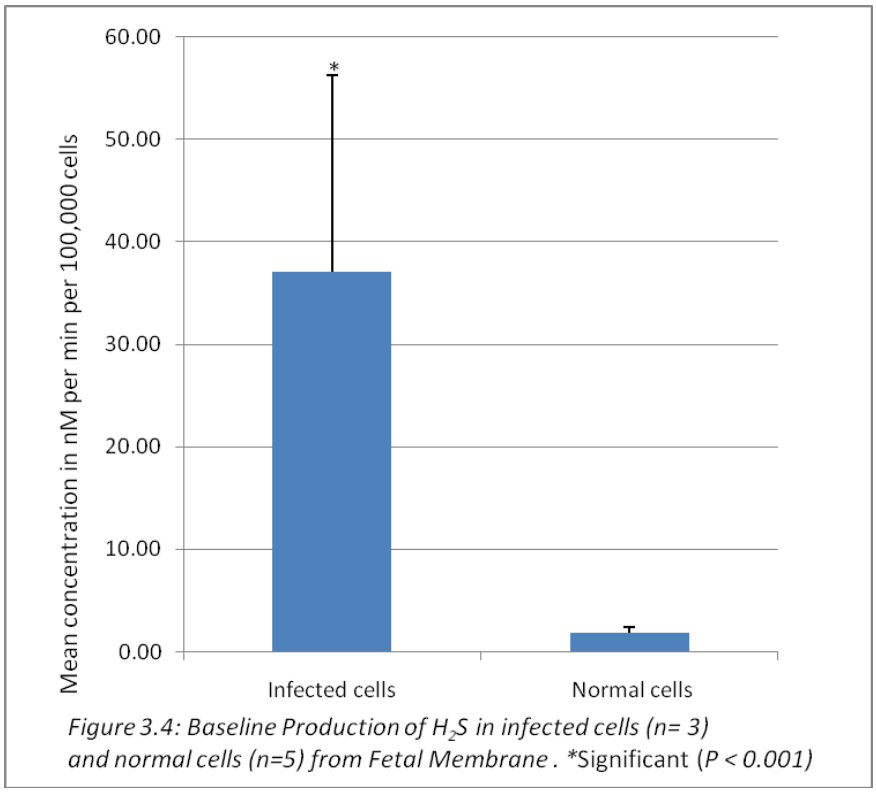

Figure 3.4: Baseline Production of H_2S in infected cells (n= 3) and normal cells (n=5) from Fetal Membrane . *Significant (*P < 0.001*)

Fetal membranes have a H_2S mean production rate in normal cells of 1.79±0.62nM/min/10^5 cells (n=5) and H_2S mean production rate in infected cells of 37.11±19.15nM/min/10^5 cells (n=3). Infected cells had a significantly (P<0.001) higher mean production rate of H_2S, in comparison normal cells.

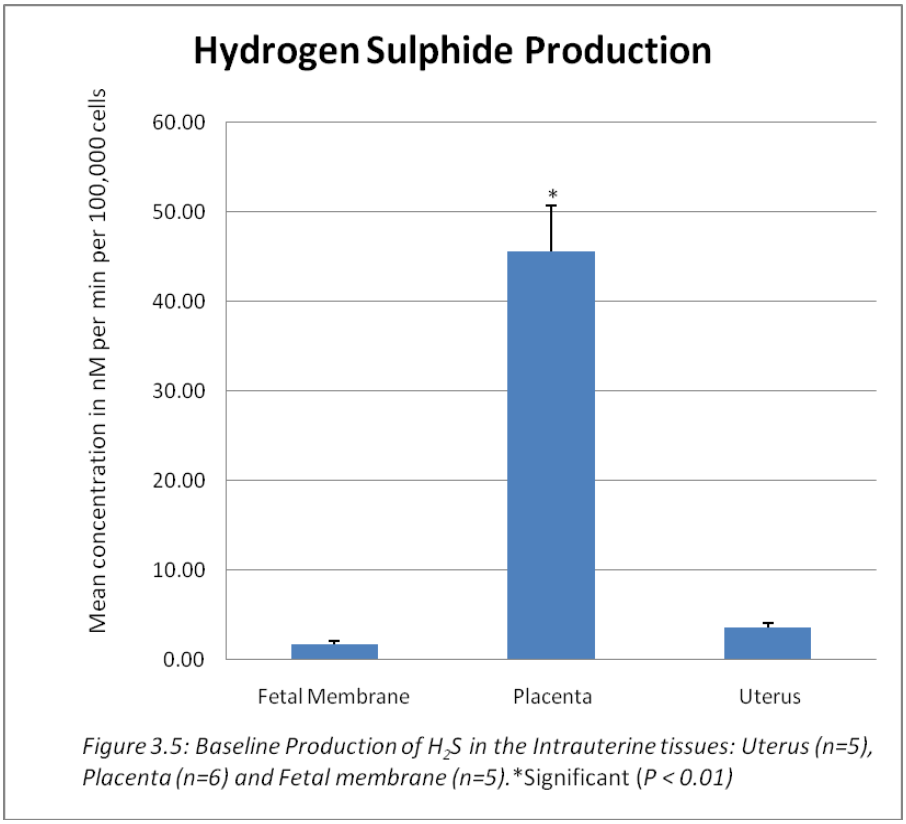

*Figure 3.5: Baseline Production of H_2S in the Intrauterine tissues: Uterus (n=5), Placenta (n=6) and Fetal membrane (n=5).*Significant ($P < 0.01$)*

Rat myometrial smooth muscle cells have a H_2S mean production rate of 3.63±1.21nM/min/10^5 cells (n=5), Chorio-decidual cells from the placenta have a H_2S mean production rate of 45.53± 12.61nM/min/10^5 cells (n=6) and cells from the fetal membranes have a H_2S mean production rate of 1.79±0.62nM/min/10^5 cells (n=5). The tissue collection was from different rats. Chorio-decidual cells from the placenta had a significantly (P<0.01) higher mean production rate of H_2S, in comparison to myometrial smooth muscle cells and cells from the fetal membranes.

3.4: Endogenous Production of H_2S with lipopolysaccharide (LPS) in Fetal Membrane

Amplitude (A)	Concentration	Cell Count	Concentration (nM per min)	Concentration (nM per min per 10^5cells)	
0.034	0.034	100,000	11.80	11.80	
0.029	0.032	100,000	10.96	10.96	+LPS
0.073	0.055	280,000	19.16	6.84	
0.051	0.043	280,000	15.06	5.38	+LPS
0.035	0.029	150,000	9.94	6.62	
0.048	0.037	150,000	12.69	8.46	+LPS
0.045	0.035	160,000	12.14	7.59	
0.040	0.032	160,000	11.04	6.90	+LPS

Table 3.6 Represents the baseline production of H_2S in amnion/chorion/decidual cells from the fetal membranes with and without LPS.

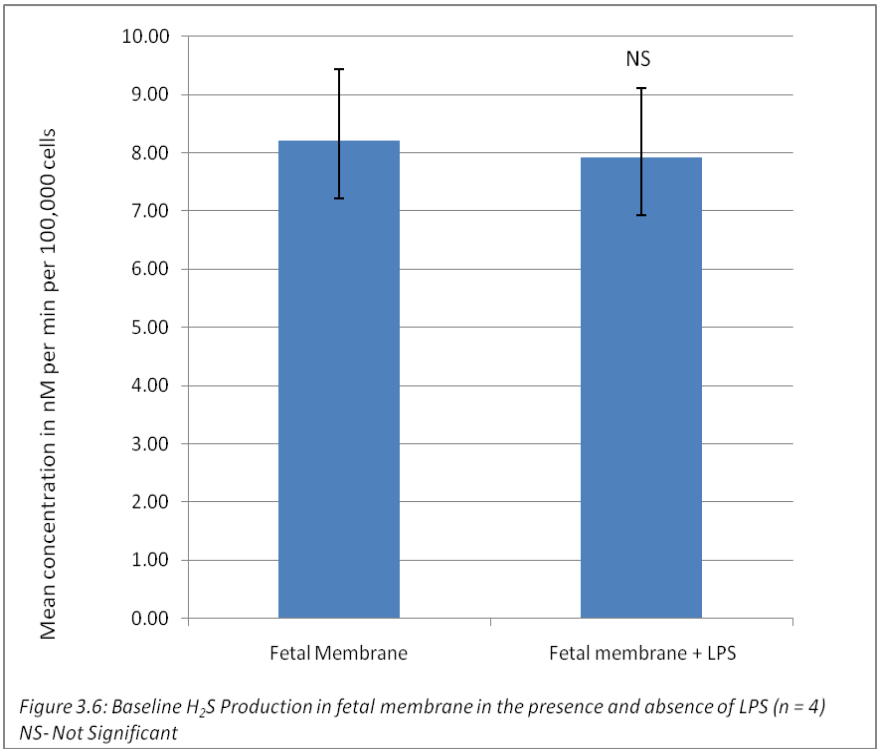

Figure 3.6: Baseline H_2S Production in fetal membrane in the presence and absence of LPS (n = 4) NS- Not Significant

Statistical analysis (T-Test) shows that there is no significant difference in H_2S production with the addition of lipopolysaccharide (control: 8.21±2.42 versus LPS treated: 7.92±2.38nM/min/10^5 cells; n=4).

Chapter Four: Discussion

Human pregnancy and labour are complicated processes and to-date there are no definite answers to how the uterus is maintained in a quiescent state or how labour is initiated. There are however a number of factors that have been found to influence the maintenance of pregnancy and initiation of labour. Premature labour is a major complication affecting pregnancy. Premature labour has been associated with multiple factors including fetal development, multiple pregnancies, previous premature deliveries, obesity, cervical incompetence, the spontaneous rupture of the membranes (PROM) and intrauterine infections (Behrman et al, 2007).

It has previously been shown that H_2S is produced by homogenates of rat uterus, placenta, fetal membranes and human placenta (Patel et al., 2009), however the exact cellular source was not known. CBS and CSE enzymes have been found widely in female reproductive tissues especially in human myometrium, amnion, chorion and placenta. This suggests that they do have some functional role in reproduction. The exact location of the CBS and CSE enzymes in these tissues is unknown as there are no published data on immunohistochemical staining of sections of reproductive tissues. One reason for this is that the early available antibodies to CBS and CSE did not work for immunohistochemistry applications. There are now much better antibodies available commercially, so hopefully this important gap in the knowledge will be filled soon.

The aim of the current study was to extract cells from the uterus, placenta and fetal membranes of pregnant rats and maintain them in culture and measure hydrogen sulphide production by these discrete cells in culture. A secondary aim was to investigate factors

that may influence the production of hydrogen sulphide in order to determine possible roles of hydrogen sulphide during pregnancy.

The cell isolation and cell culture technique, involves the use of digesting/ dissociating enzymes to break down the connective tissue that hold the cells together. It is a common technique and for this research the enzymes used were trypsin, DNAse and collagenase. The cell culture technique is an aseptic technique and care was taken to maintain this at all times. However, there was a problem with a persistent infective agent in the cell cultures which eluded identification, despite the use of penicillin and streptomycin. The capture and measurement of H_2S production was adapted from Patel et al (2009). The methylene blue assay was used to measure endogenous production of H_2S. The assay was reproducible and gave linearity (R^2) of > 0.98.

Patel et al (2009) has shown that H_2S is produced in homogenised rat fetal membrane, placenta and uterus and human placenta. It was shown that CBS and CSE are present in human myometrium, amnion, chorion and placenta. Homogenates did not give a definite identity to the source of H_2S production as it has been shown that CSE is expressed in the walls of blood vessels and so any large blood vessels in the tissue sample could be a source of CSE. The current research was therefore done using primary cell culture incubations of myometrial smooth muscle cells, chorio-decidual cells from the placenta and amnion/chorion/decidual cells from fetal membranes from pregnant rats. This showed that rat chorion, decidual, amnion and myometrial smooth muscle cells can produce H_2S in culture. It is difficult to compare the results from this publication with the current study. In Patel et al (2009) H_2S production was measured in nM/min/g however in this research H_2S production was measured in nM/min/10^5cells.

The highest production rate of H_2S was from chorion-decidual cells from the placenta. The mean production rate of these cells was 45.53±12.61nM/min/10^5cells (n=6). The placenta is a highly metabolic organ which produces many hormones and this could be the reason for the high production rate. The high production rate may also be related to its possible function in maintaining pregnancy. It is possible that the H_2S produced in placenta circulates into surrounding tissues causing vasodilation of placental blood vessels or maintaining myometrial quiescence. Research shows that H_2S is a vasodilator and is involved in the regulation of blood pressure (Wang, 2008). H_2S could be involved in the local control of blood flow, particularly in the placenta; however this still has to be researched further as there are currently no published studies in this area using human placental blood vessels.

There has been previous research done by Hayden et al, (1990) showing that H_2S administered externally prolongs the duration of delivery in rats. Sidhu et al (2001) also showed that L-cysteine relaxes pregnant rat uterus in vitro. This means that H_2S could act locally in myometrial smooth muscle cells thereby maintaining uterine quiescence. H_2S donors have been shown to inhibit spontaneous uterine contractions in rats in vitro (Sidhu et al., 2008). So far, there are no data on effects on human myometrium. Uterine contractions could be inhibited by an upregulation of CBS and CSE in pregnancy which causes production of H_2S in the myometrium and placenta.

H_2S may have a role as an inflammatory mediator in the reproductive system however this role is still unclear. There is good evidence that the initiation of parturition in humans involves inflammation (Norman et al, 2007). Various studies have shown that nitric oxide has a function as an inflammatory mediator, particularly in the cervix at term (Norman et al,

2007). Previous studies have shown that intrauterine infections may be a risk factor in premature labour. It is suggested that premature labour is caused by the activation of the innate immune system and inflammation triggered by intrauterine bacteria.

Lipopolysaccharide (LPS) is a lipid and polysaccharide joined by a covalent bond. It is a major component found on the outer cell wall of certain bacteria. The presence of LPS triggers an innate immune response to infection. LPS mimics the presence of bacteria, therefore the addition of LPS in fetal membrane culture mimics possible intrauterine infection. However, in this study there was no significant difference between H_2S production rate in the fetal membrane cells with the addition of lipopolysaccharide. This means that H_2S production does not increase when there is a bacterial infection in the fetal membrane. Therefore increased H_2S production does not appear to act as an inflammatory mediator in the fetal membranes, whereas this was found to be the case for nitric oxide (Seyffarth et al., 2004). .

In the process of this research there were various complications with infections in the cell cultures. The infection was prevalent even though Penicillin Streptomycin (PenStrep) and Nystatin where used. The measurement of H_2S production was still calculated even for the cell cultures that appeared infected. The H_2S production of the infected culture where compared with those of non-infected culture and there was a significant difference. The H_2S production in the infected cell culture was higher than that of the non-infected culture. The higher rate of H_2S production by the infected cells could be due to the infecting agent triggering more production from the uterine cells, whereas LPS alone did not do this. However it is also possible that the infective agent could breakdown L-cysteine to produce the H_2S directly. In laboratory conditions where LPS was used the outcome was that there

was no significant increase in the production of H_2S from the fetal membrane cells, however in the cases of the infected cells the production was significantly higher, this means that the most likely explanation is that the infective agent was breaking down the L-cysteine to give H_2S. More controlled research will need to be carried out to determine the actual role of bacterial infections on H_2S production in the intrauterine cells.

Although it has been stated that H_2S could be involved in reproduction however one research has shown that this may not be accurate as CSE -/- knockout mice are fertile and give birth normally (Wang et al, 2008). However this may not be the same for human as it is possible that there are differences in species.

Srilatha et al (2009) has shown that H_2S relaxes the smooth muscle partially via cAMP, NO/cGMP and K^+_{ATP} channels. This was found out by the use of specific inhibitors of signalling pathways.

Further work

Further work must be done to understand the production of H_2S from intrauterine tissues. It is important to investigate the enzymes that are involved in this production. Immunohistochemical staining, Western blotting and RT-PCR for mRNA for both CBS and CSE enzymes need to be carried out. Completing these research will assist to determine the location of production of H_2S and possible roles. Research also needs to be carried out to investigate factors which affect the expression of CBS and CSE.

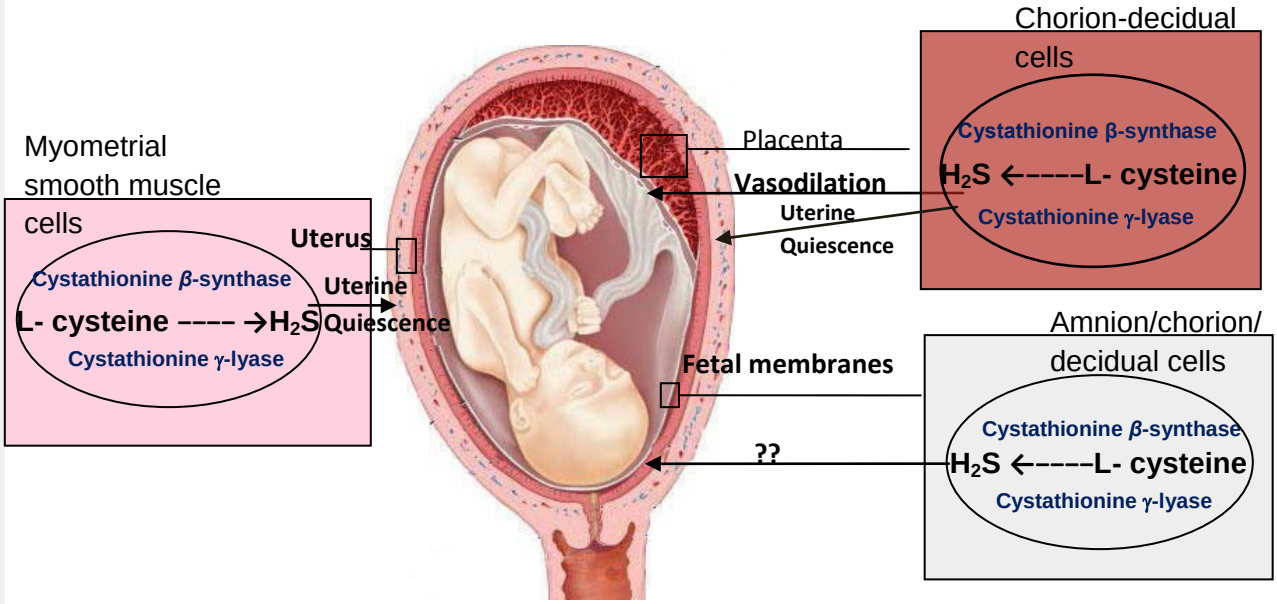

Figure 5.1 shows a model of the possible roles of H_2S in the female reproductive tract in pregnancy.

The diagram above is a model of the production of hydrogen sulphide from myometrial smooth muscle cells, chorio-decidual cells from the placenta and amnion/chorion/decidual cells from fetal membranes of pregnant intrauterine tissues and its possible roles. The model shows that hydrogen sulphide produced in chorion-decidual cells of placenta and myometrial smooth muscle cells could be involved in maintaining myometrial quiescence. The function of H_2S produced in amnion/chorion/decidual cells of the fetal membranes is not yet known.

In conclusion this research has shown that myometrial smooth muscle cells, chorio-decidual cells from the placenta and amnion/chorion/decidual cells from fetal membranes can convert L-cysteine into H_2S. These findings suggest that H_2S could be involved in

uterine quiescence and vasodilation leading to regulation of local blood flow. However this research was done using rat uterus, placenta and fetal membrane. It is therefore necessary to repeat the research using human uterus, placenta and fetal membrane as there might be species-dependent differences in the result. It is then essential that further research is carried out to confirm/determine the role of H_2S within the intrauterine tissue and the mechanisms.

Despite several interventions designed to inhibit preterm labour and prolong pregnancy, the frequency of preterm birth is still a great problem worldwide. If the roles of H_2S within pregnancy are established it may prove to be a way forward in preventing and/or treating premature labour.

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